Exhibit C

Crossreactive B cells are present during a primary but not secondary response in BALB/c mice expressing a bcl-2 transgene

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Abstract

While it is clear that multiple genetic factors lead to autoimmune diseases such as systemic lupus erythematosus (SLE), it appears that an environmental stimulus is also required to trigger the disease in susceptible individuals. We have previously demonstrated that B cells making crossreactive antibodies that bind to both phosphorylcholine (PC), a component of pneumococcal cell wall polysaccharide, and double stranded DNA (dsDNA) can be found in BALB/c mice immunized with PC coupled to a protein carrier. While these B cells are normally eliminated in vivo by apoptosis, they can be recovered ex vivo by fusion with a cell line overexpressing the anti-apoptotic gene bcl-2. This observation led us to ask whether in vivo expression of bcl-2 might abrogate immunologic tolerance during an ongoing immune response. In the present study, we have examined BALB/c mice that constitutively express a bcl-2 transgene in the B cell compartment. Bcl-2 transgenic BALB/c mice have an expanded B cell number, but display no evidence of anti-dsDNA antibodies in the serum even following immunization with PC coupled to a protein carrier. Crossreactive anti-DNA, anti-PC B cells can be recovered by hybridoma technology late in the primary response, but do not appear in the memory B cell compartment. Thus, in vivo expression of bcl-2 can rescue B cell autoreactivity in the primary immune response, but is not sufficient for activation of these B cells or for their maintenance in the memory compartment. © 1999 Elsevier Science Ltd. All rights reserved.

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1. Introduction

Systemic lupus erythematosus is an autoimmune disease characterized by the production of antibodies against a variety of nuclear antigens, including dsDNA. Regulation of autoreactive B cells is thought to occur at multiple developmental checkpoints, with apoptosis being an important regulatory mechanism. B cells arising in the bone marrow that bind self antigen with high avidity are either deleted (Hartley et al.,

1991; Erikson et al., 1991; Spatz et al., 1997; Nemazee and Burki, 1989) or undergo additional immunoglobulin gene rearrangements to generate non-autoreactive specificities, a process termed receptor editing (Tiegs et al., 1993; Gay et al., 1993). B cells that experience a lower receptor occupancy with self antigen in the bone marrow escape deletion and are allowed to circulate in a state of clonal anergy (Goodnow et al., 1988; Shlomchik et al., 1993; Fulcher and Basten, 1994) in which they are functionally silent and have a shortened life span. Anergy has been shown to be reversible with lipopolysaccharide (LPS) stimulation (Spatz et al., 1997; Goodnow et al., 1991), suggesting that autoreactive B cells might be available for recruitment into an ongoing immune response. The implications for autoimmune disease are that genetic alterations affecting apoptotic pathways might predispose toward the development of autoreactivity.

472

In addition to a genetic predisposition, there may also be a need for an environmental trigger prior to the development of autoimmune disease. This is suggested by the fact that autoimmune strains of mice may display a reduced onset of disease when kept under germfree conditions (Unni et al., 1975; Murakami et al., 1997). Environmental antigen has been proposed as one possible trigger, with activation of autoreactive B cells resulting from crossreactivity between foreign and self antigen. Crossreactivity between phosphorylcholine (PC) and double stranded (dsDNA) was first demonstrated in analyses of the antigenic fine specificity of anti-DNA antibodies (Andrzejewski et al., 1981; Limpanasithikul et al., 1995). Subsequently, it was shown that crossreactive B cells arise during the response to immunization with PC in BALB/c mice. These cells are thought to undergo apoptosis, as dual specificity antibodies are not present in serum and crossreactive B cells can be rescued only by fusion with the NSO^{bcl-2} cell line that constitutively expresses bcl-2 (Ray et al., 1996; Ray and Diamond, 1994). To ask how autoreactivity is regulated in vivo during either the primary or secondary response to foreign antigen, we elected to study the B cell response to immunization with PC-carrier in bcl-2 transgenic Eμbcl-2-22BALB/c mice (Strasser et al., 1991).

We show here that anti-dsDNA antibodies were not detectable in the serum of naive bcl-2 transgenic BALB/c mice. After immunization with PC, however, crossreactive B cells were readily isolated by somatic cell fusion during the late primary immune response to PC. Interestingly, none were identified during the secondary immune response. These data suggest that crossreactive B cells are less stringently controlled during the primary response than during the secondary response.

2. Materials and methods

2.1. Animals and immunizations

BALB/c transgenic Eμbcl-2.22 mice (Strasser et al., 1991) were generously provided by J. Thorbecke at New York University with permission from A. Harris at the Walter and Eliza Hall Institute, Melbourne, Australia. Animals were housed in a specific pathogen-free facility and were backcrossed to the BALB/c background for six to nine generations. Mice were primed intraperitoneally with 100 μg keyhole limpet hemocyanin (KLH) in 100 μl of Complete Freund's Adjuvant (CFA):saline (1:1), and 1-2 weeks later were given an intraperitoneal injection of 50 μg of PC-KLH in 100 μl saline. For secondary immunizations, an additional intraperitoneal injection of 50 μg PC-KLH in saline was given after 2 months.

2.2. Generation of hybridomas

Splenocytes were handled on ice in order to reduce cell loss by apoptosis. Fusions were performed as previously described (Fazekas de St Groth and Scheidegger, 1980). Briefly, hybridomas were generated using 50% PEG 4000 to fuse splenocytes with the NSO fusion partner, followed by selection in HAT medium. All cell lines were cloned either in soft agarose or by limiting dilution. Immunized wild type mice typically yielded 1×10^8 splenocytes, while bcl-2 transgenic mice yielded two to four times as many cells.

2.3. ELISAs

Antibodies in hybridoma supernatants were quantitated by ELISA using isotype-matched standards and the concentration of antibodies was normalized prior to use in antigen-specific assays. All blocking steps and serum antibody dilutions were performed with 1% BSA in PBS. Anti-PC or anti-dsDNA antibodies were detected with isotype-specific secondary antibodies coupled to alkaline phosphatase (Fisher Biotech., Pittsburgh, PA and Southern Biotech, Birmingham, AL). The plates were developed using the Sigma 104 substrate (Sigma Chemical Co.) and OD was measured at 405 nm using a Titertek Multiscan Plus plate reader (Ealabs, Finland).

PC binding assays were performed on Falcon 3915 ELISA plates (Becton Dickinson, Lincoln Park, NJ) coated with 2 μg/well of PC-BSA. Samples were screened for binding to PC-BSA instead of PC-KLH in order to avoid detection of antibodies against the protein carrier KLH. This assay can detect even low affinity anti-PC antibodies (Ray et al., 1996). DNA binding assays were performed on Immulon II (Dynatech, Chantilly, VA) ELISA plates coated with 10 μg/well of calf thymus dsDNA which has been filtered through a 0.2 μm methyl-cellulose membrane (Millipore Corporation, Bedford, MA) to remove single-stranded DNA. Inhibition ELISAs were performed by incubating antibody samples for 20 min at room temperature with soluble PC-chloride (Sigma Chemical Co., St Louis, MO) prior to performing the direct binding assay described above. Enhancing ELISAs were performed using a protocol adapted from Valadon and Scharff (1996) in which hybridoma supernatants were co-incubated with serially titrated amounts of alkaline phosphatase-labeled secondary antibodies for 3 h at 37°C before developing the assay.
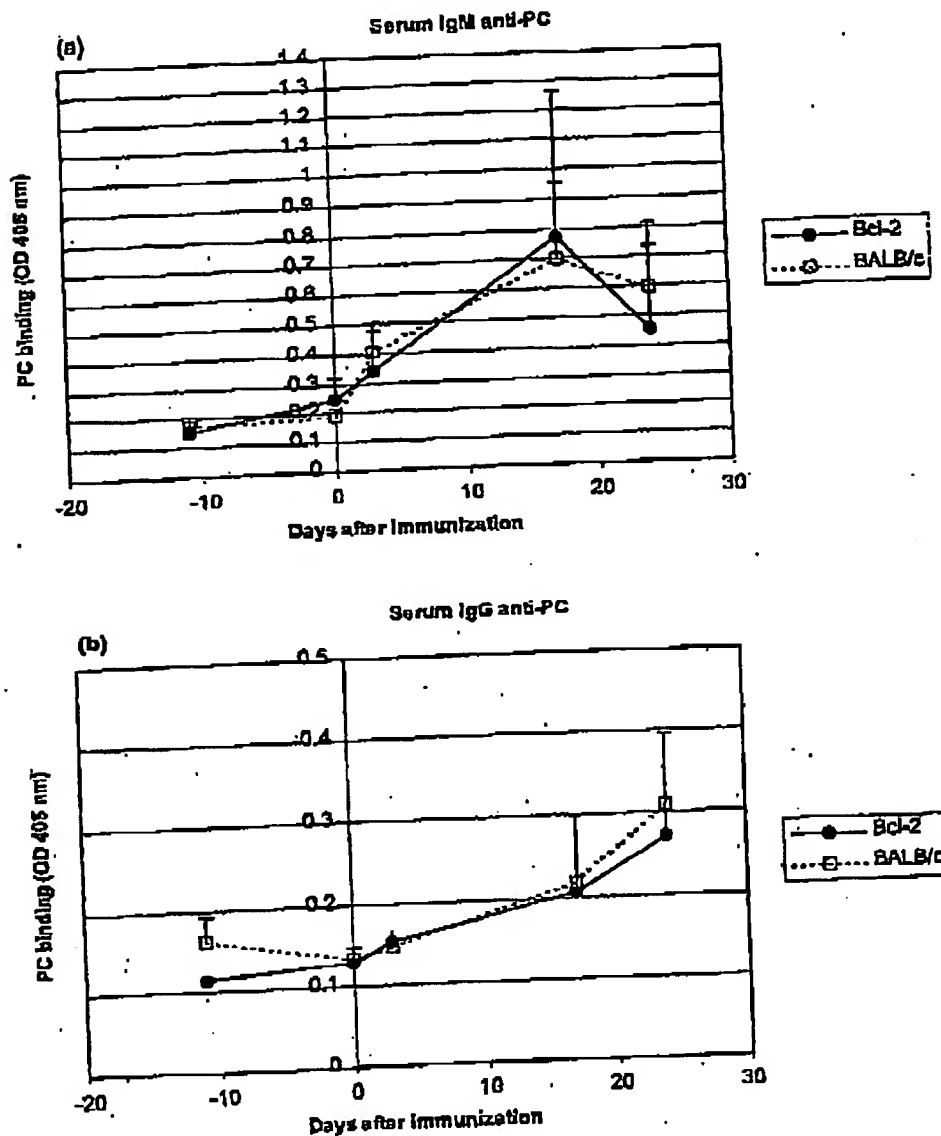
473

Fig. 1. Serum anti-PC responses in immunized bcl-2 transgenic mice and non-transgenic littermates. Serum was diluted 1:1000 for detection of IgG antibodies and 1:5000 for detection of IgM antibodies. IgM (a) and IgG (b) anti-PC response measured on PC-BSA-coated ELISA plates. Each line represents an average from 3 mice, and error bars indicate one standard deviation from the mean.

2.4. RT-PCR and sequencing of antibody mRNA

RNA was made from each cell line using the Ultraspec kit (Biotecx, Houston, TX). cDNA was generated using Superscript II reverse transcriptase (GibcoBRL, Gaithersburg, MD) according to the manufacturer's directions, and antibody genes were amplified by PCR using Vent polymerase (New England Biolabs, Beverly, MA) in commercially provided buffer plus 1 mM $MgSO_4$ and 1% DMSO. Amplifications were performed using antibody heavy

or light chain degenerate primers on the 5' end and constant region primers on the 3' end (κ light chain, TGGATGGTGGGAAGATG; μ heavy chain, GCAGGAGACGAGGGGGA; γ heavy chain, GGGGGCAGTGGATAGAC.) When necessary, amplified DNA bands were gel-purified using the Qiaex II kit (Qiagen, Santa Clarita, CA). DNA from PCR reactions was purified for sequencing using the Qiaquick kit (Qiagen) and resuspended in distilled water.

Automated sequencing on an ABI 377 (Applied

474

Table 1
IgM anti-PC hybridomas (N.D.= not determined)

Fusion	IgM clones	V$_H$	V$_H$ mutations
Primary, day 3	F14.2F6	S107(V1)	1/284
	F14.3B7	S107(V1)-DFL16.1	0/299
	F14.3E5	S107(V1)-DFL16.1	0/299
	F14.7C7	S107(V1)-DFL16.1	0/298
	F14.14A2	S107(V1)-DFL16.1	0/281
	F14.3C8		
	F14.4D5		
	F14.6A12		
	F14.6G9		
	F14.7A2		
	F14.14A12		
	F14.15A8		
	F14.17G6		
	F14.18H11		
	F14.19G12		
	F3.11E9		
Primary, day 10	S1.7F4	S107(V1)-DFL16.1-J2	0/279
	S1.19C8	J558(V50)-DSP2.3-J3	18/269
	S1.26F11	S107(V1)-DFL16.1-J1	7/278
	F4.27F7	S107+	
	S1.4B2	N.D.	
	S1.19C6	J558(V2-12)-DLF16.2-J3	0/268[a]
	S1.23C5	V7183(14.29)-DFL16.1-J2	0/272
	S1.27E7	J558(H10)-Q52-J4	0/270[a]
	R13.16G2	J558-DFL16.2-J4	0/269[b]

[a] Mutations present in D$_H$ segment.
[b] Compared with an unpublished antibody (GenBand № AF007229).

Biosystems Inc., Foster City, CA) was performed at the Sequencing Facility of the Albert Einstein College of Medicine. Analysis of sequences was performed with Genetics Comparison Group (GCG) software, version 8.0 (Madison, WI).

3. Results

3.1. Bcl-2 transgenic mice mount normal anti-PC serum antibody responses

BALB/c mice constitutively expressing bcl-2 in B cells have increased numbers of circulating B cells and enlarged spleens but no increase in serum titers of anti-PC or anti-DNA antibody even at 6 months of age. Following immunization with PC-KLH, bcl-2 transgenic mice mounted primary and secondary serum IgM and IgG anti-PC responses that were comparable to non-transgenic littermate controls (Fig. 1). The titers of serum anti-dsDNA antibodies did not rise following primary or secondary PC immunization in either bcl-2 transgenic or control mice (data not shown).

3.2. Three days after PC immunization the primary B cell response is dominated by the canonical T15 antibody

The serum response to PC in BALB/c mice is dominated by T15 antibodies, encoded by the S107 V$_1$ heavy chain gene and the V$_K$22 light chain gene (Crews et al., 1981). In order to see if overexpression of bcl-2 altered B cell selection, we characterized splenic B cells from PC-KLH immunized bcl-2 transgenic mice. B cell fusions were performed 3 days after primary immunization with PC-KLH. Twenty-nine PC binding lines were obtained; all produced IgM antibodies. RNA dot blot analysis showed that 24/29 (83%) of the lines expressed a S107 V$_H$ gene. Sixteen lines were cloned. Five S107 V$_H$ expressing clones were randomly selected for sequencing (summarized in Table 1). Four expressed that S107(V1)-DFL16.1 heavy chain antibody rearrangement present in canonical anti-PC antibodies, while one clone (F14.2F6) expressed the S107(V1) gene in association with a non-canonical D region (GenBank accession numbers F044224-AF044230). Only one mutation was found in these five heavy chains, for a low overall mutation frequency of less than 0.1% (1/1461 nucleotides) as would be expected early in a primary antibody response. Also similar to the response in wild type BALB/c mice, the light chain sequences from these five clones were encoded by unmutated canonical V$_K$22 genes.

Binding of most antibodies to PC was comparable to PC2μ, a canonical S107(V1)V$_H$ and V$_K$22 encoded anti-PC antibody (Fig. 2), consistent with the observation that most antibodies had the same VDJ and VJ rearrangement as PC2μ, without somatic mutation. Like PC2μ, none bound to dsDNA, demonstrating that by day 3 after immunization, there was primarily recruitment of canonical non-crossreactive anti-PC B cells in bcl-2 transgenic mice and no detectable alteration in B cell selection (Table 1).

3.3. Ten days after PC immunization, the primary response includes crossreactive anti-PC, anti-dsDNA B cells

To determine whether somatic mutation might lead to the generation of crossreactive anti-PC, anti-DNA B cells, we obtained nine IgM anti-PC clones 10 days after primary immunization with PC-KLH. Six of these were crossreactive with dsDNA. These crossreactive clones were not uniformly of high affinity for PC; only three clones (S1.4B2, S1.19C6, S1.27E7) displayed binding to PC-BSA which was comparable to that of the canonical PC2μ antibody. Two of the six, S1.4B2 and S.27E7, displayed binding to dsDNA comparable to that of hybridoma F100b10.33, a crossreactive IgM
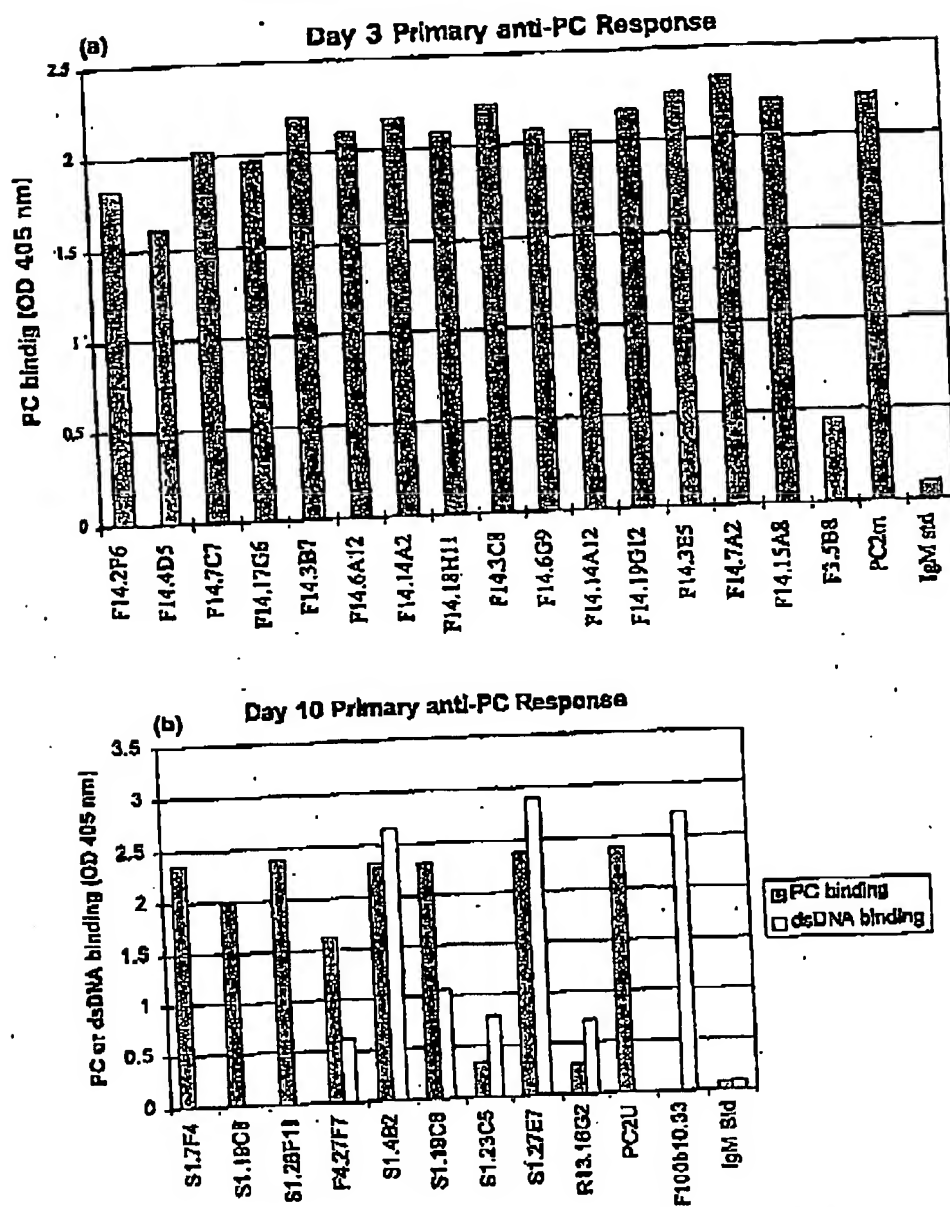
475

Day 3 Primary anti-PC Response

Day 10 Primary anti-PC Response

Fig. 2. (a) Anti-PC clones from day 3 after primary immunization. Hybridoma supernatants were normalized to an antibody concentration of 5 µg/ml and PC binding was measured by ELISA. PC2µ, a canonical anti-PC antibody, was used as a positive control, and a non-binding mouse IgM standard was used as a negative control; (b) crossreactivity of anti-PC clones from day 10 after primary immunization. Hybridoma supernatants were normalized to an antibody concentration of 1.25 µg/ml and PC (dark bars) or dsDNA (light bars) binding was measured by ELISA. PC binding was compared to PC2µ and dsDNA binding was compared to F100b10.33 a DNA binding antibody (14). An isotype-matched, non-binding mouse IGM standard was used as a negative control).

anti-PC, anti-dsDNA clone that has been previously shown to deposit in renal glomeruli (Ray et al., 1996) (Fig. 2b).

RNA dot blot analysis and sequencing of antibody heavy chains revealed that only three clones (S1.7F4, S1.26F11, and F4.27F7) expressed the S107(V1) gene;

the other 6 clones used V_H genes derived from the J558 and V7183 families (GenBank accession numbers AF044232-AF044239). Thus, by day 10 following immunization, there was evidence of recruitment of non-canonical, potentially pathogenic, crossreactive B cells into the anti-PC response. Sequence analysis of the

476

Table 2
IgG anti-PC IgG hybridomas; PC binding values, using 2.5 μg/ml of antibody, are given as ELISA absorbances

Fusion	IgG clones	PC binding	V_H genes	V_H mutations	V_L
Controls	Anti-PC IgG1	0.602			
	Anti-PC IgG2a	1.226			
	Anti-PC IgG2b	0.973			
	IgG2b standard	0.199			
Secondary, day 3	F1.2B10	2.305	S107(V1)	9/215	V_K
	F1.3C4	2.264	V7183(V283)	19/220	V_1
Secondary, day 10	F2.3D12	0.353	S107(V1)	5/222	V_K
	F5.18B12	0.348			V_x
	F5.20C4	1.010			V_K
	F5.26E11	1.680			V_K
	F10.15H3	0.349			V_K
	F10.16H5	0.555			V_K
	F10.30F6	0.301			V_K
	F10.35F9	1.153			V_K

crossreactive antibodies showed that the V_H segments of these antibodies were unmutated: however, the D

segments were not completely homologous to known germline sequences, suggesting that these antibodies may be somatically mutated (summarized in Table 1).
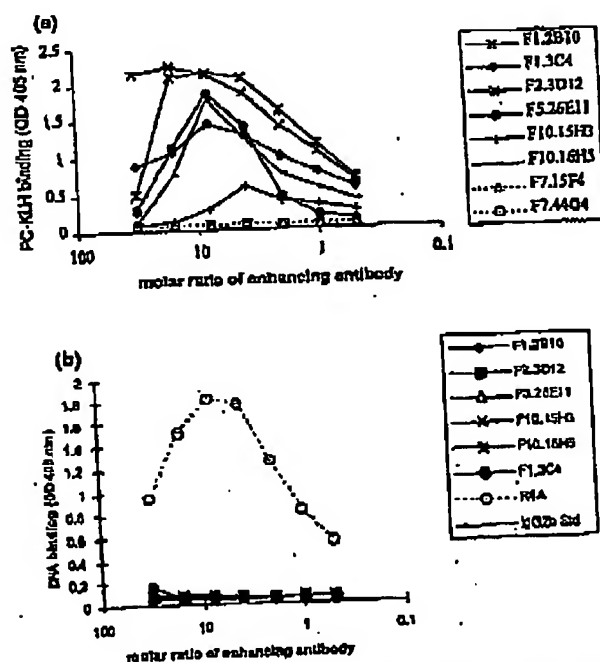

Fig. 3. Enhancing ELISA of IgG clones. Normalized antibody supernatants (5 μg/ml) were co-incubated with serial titrations of labeled secondary antibody (enhancing antibody). Horizontal axis represents the molar ratio of enhancing antibody to sample. Enhancement of PC or dsDNA binding was measured at a time point when ODs on the non-enhancing ELISA were barely detectable. Control hybridoma supernatants from F7.15F4 (λ) and F7.44G4 (κ) contain IgG2b antibodies, and bind to dsDNA but do not bind PC (dashed lines). (a) Enhancement of PC binding; (b) enhancement of dsDNA binding compared to R4A, an anti-dsDNA IgG2b antibody, and non-binding IgG2b standard.

3.4. Crossreactivity to dsDNA disappears after secondary immunization

To determine whether crossreactive B cells from Eμbcl-2-22 mice would be present in the memory B cell compartment, hybridomas were derived from mice given a PC-KLH boost (Table 2); ten IgG anti-PC clones were obtained; none was crossreactive with dsDNA (data not shown). Only two clones, F1.2B10 and F2.3D12, were encoded by the S107(V1) heavy chain gene, demonstrating that non-canonical, anti-PC B cells do transit into the memory B cell compartment, although those that crossreact with dsDNA do not. Three IgG anti-PC antibodies were selected for sequence analysis. All contained multiple somatic mutations in the heavy chain genes, consistent with a secondary immune response (Table 2).

Because IgM antibodies are pentamers and IgG antibodies are monomers, IgM antibodies might be more easily detected in an antigen binding assay. To ascertain whether the lack of dsDNA binding by the IgG anti-PC antibodies reflected the reduced avidity of IgG compared to IgM, we used goat anti-mouse light IgG compared to IgM, we used goat anti-mouse light chain antibody to crosslink the IgG antibodies. Formation of antibody complexes in this manner artificially enhances the avidity of binding. As can be seen from Fig. 3, an appropriate ratio of enhancing antibody dramatically increased binding to PC-KLH. When the same concentration of crosslinking antibody was used in a dsDNA ELISA (Fig. 3), no dsDNA binding was observed, demonstrating that the lack of

crossreactivity cannot be explained by a reduced avidity for antigen.

4. Discussion

Current theories of autoimmunity often posit a critical role for molecular mimicry in the activation of autoreactive T and B cells (Zhao et al., 1998; Gross et al., 1998). Crossreactive T and B cells recognizing both a foreign antigen and a self antigen can be activated when the host encounters foreign antigen in an immunogenic context. There is increasing evidence that self antigen may then perpetuate the activation of crossreactive lymphoid cells that were primed on foreign antigen. We have been studying the antibody response to PC, a component of bacterial polysaccharide, in BALB/c mice. While the serum response following primary immunization is dominated by antibodies of the T15 idiotype encoded by a single heavy chain variable region gene and a restricted number of light chain variable region genes, we have previously shown that the spleen of immunized mice contain B cells making non-canonical anti-PC antibodies (Ray et al., 1996). These antibodies are encoded by a variety of V_H and V_L gene segments and a high percentage, approximately 40%, crossreact with dsDNA. These cells normally undergo apoptosis as they can only be rescued by somatic cell fusion with NSO^{bcl-2}, a B cell myeloma line that constitutively overexpresses bcl-2.

Given the observation that crossreactive B cells can be rescued from apoptosis by fusion with a bcl-2 expressing cell, we asked whether in vivo overexpression of bcl-2 might lead to autoantibody secretion and the recruitment of autoreactive cells into the secondary response. Bcl-2 has been shown to prolong survival of autoreactive cells, but these cells often remain in an inactive state. In mice expressing both an anti-hen egg lysozyme antibody and membrane bound hen egg lysozyme as transgenes, autoreactive B cells routinely undergo deletion and are not found in peripheral lymphoid organs (Hartley et al., 1993). When the autoreactive B cells also express a bcl-2 transgene, the cells exit the bone marrow, but survive for only a short time and in a 'developmentally arrested' state. Similarly, in mice expressing both an anti-K^b antibody and K^b in the periphery, B cell expression of bcl-2 leads to prolonged B cell survival but no activation of antibody secretion (Lang et al., 1997). In these models, however, there was no antigen presentation and no antigen specific T cell help available to activate the autoreactive B cells. Thus, the lack of B cell activation might have resulted from a lack of necessary co-stimulation.

In the study described here, we asked whether autoreactive B cells rescued from apoptosis by a bcl-2

transgene could be activated in vivo by molecular mimicry when both foreign antigen and antigen activated T cells were present. B cells that crossreact with both foreign and self antigen may represent a link between microbial infection and autoimmune disease (Kuo et al., 1996). PC, a component of bacterial polysaccharide, is known to have structural features in common with dsDNA and many anti-PC antibodies crossreact with DNA. Furthermore, immunization of BALB/c mice with PC-carrier has previously been shown to augment a population of tolerized crossreactive B cells (Ray et al., 1996). Thus, we studied BALB/c mice immunized with PC-carrier to determine if molecular mimicry can activate anti-PC, anti-DNA bcl-2 transgenic B cells generated during primary or secondary anti-PC antibody responses.

In both wild type and bcl-2 transgenic mice, there was no constitutive titer of anti-DNA antibody and no rise in anti-DNA antibody titer following primary and secondary immunization. During the late primary response, however, it was possible to isolate crossreactive B cells as hybridomas from bcl-2 transgenic mice. Sequence analysis of the crossreactive antibodies demonstrated that many were encoded by unmutated V_H genes, suggesting that the bcl-2 transgene may allow crossreactive B cells to exit the bone marrow. This is consistent with previous studies in autoantibody transgenic mice, as discussed above (Hartwell et al., 1995; Lang et al., 1997). The absence of crossreactive B cells early in the response may reflect more effective competition for antigen by the canonical T15 anti-PC B cells, or a higher frequency of this clone in immunized mice.

Despite the presence of crossreactive B cells at day 10 following immunization, no crossreactive IgG producing B cells were obtained from splenocytes during the secondary response. This observation could reflect the force of either positive selection of non-autoreactive clones, or negative selection against autoreactive clones. It is important to note that at least three crossreactive B cell clones, S1.4B2, S1.1AC6, S1.27E7, bound to PC as well as the canonical S107V1 encoded antibody, suggesting that these crossreactive B cells would compete effectively for antigen and undergo positive selection. Because affinity maturation is not a characteristic of the anti-PC response, and there are no data to suggest that mutation in the late primary or secondary response leads to increased PC binding concomitant with a loss of DNA binding, we believe that the absence of crossreactivity in the secondary response suggests that a tolerance checkpoint exists either immediately before or within the memory compartment. Cyster et al. (1994) have shown that anergized B cells adoptively transferred into non-anergized hosts are unable to enter B cell follicles (Goodnow, 1996). A similar fate appears to exist for the crossreac-

478

tive cells that were present at day 10 of the primary response, such that they were not recruited into a secondary response to PC.

Hande et al. (1998) have demonstrated that A/J mice expressing a different bcl-2 transgene and immunized with azophenylarsonate display crossreactive anti-arsonate, anti-DNA B cells in the secondary immune response. The apparent contradiction to the observations reported here has several possible explanations. The crossreactive B cells in the secondary anti-arsonate response may be subject to very strong positive selection. They have an affinity for arsonate that is at least one log higher than the affinity of the B cells found in the primary response. Perhaps, this increased affinity helps mediate positive selection and explains the survival of crossreactive B cells in the anti-arsonate memory response. It is also possible that because of differences in the bcl-2 transgene used in the mice studied by Hande et al. (1998), there may be a different level of expression or timing of expression of the bcl-2 transgene in the arsonate immunized mice that permits crossreactive cells to enter a memory compartment (Chleq-Deschamps et al., 1993; Haldar et al., 1995; Oltvai et al., 1993; Reed, 1997). Finally, and perhaps most likely, the difference in the survival of crossreactive B cells in the secondary response may reflect differences in the genetic backgrounds of the mice. For example, the bcl-2 transgene in the SJL mouse leads to autoimmunity and lupus-like disease. The same transgene in BALB/c mice, like in C57BL/6 mice, does not lead to autoantibody titers or nephritis (Strasser et al., 1992).

Our data suggest that crossreactive B cells arising in BALB/c mice during an ongoing response to foreign antigen are tolerized following antigenic stimulation and are not maintained for recruitment into a secondary immune response. Based on this observation, we would speculate that the regulation of naive B cells may be less stringent than the regulation of antigen activated B cells. Thus, crossreactive B cells from the naive B cell repertoire are present in a primary immune response but are not maintained in the secondary response. The preferential survival of autoreactive B cells from the naive B cell repertoire would permit existence of a naive B cell repertoire capable of netting the largest number of antigens, while the more stringent selection of memory B cells would eliminate the development of potentially pathogenic autoreactivity. B cells about to secrete IgG antibody or enter a long lived memory compartment appear to be tolerized, while resting naive autoreactive B cells are activated. Thus, it appears that regulation serves to maximize the repertoire, while still protecting against the presence of pathogenic autoantibodies in the serum response.

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